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Firefly Luciferase ATP Assay Development for Monitoring Bacterial Concentrations in Water Supplies

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FIREFLY LUCIFERASE ATP ASSAY DEVELOPMENT FOR MONITORING BACTERIAL CONCENTRATIONS IN WATER SUPPLIES

by

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This study was conducted
in cooperation with the
National Aeronautics and Space Administration
Goddard Space Flight Center
Greenbelt, Maryland 20771

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16. ABSTRACT

This research program was initiated to develop a rapid, automatable system for measuring total viable microorganisms in potable drinking water supplies using the firefly luciferase ATP assay.

The assay was adapted to an automatable flow system that provided comparable sensitivity with established methodology (10^5 bacteria per ml) and assays in less than 2 minutes. Quality controls for required reagents were established. To achieve the sensitivity necessary for bacterial measurements in water, the sample must be concentrated prior to assay. Filtration systems were evaluated for ability to concentrate bacteria from large sample volumes rapidly, efficiently, and without damage to the organisms.

Results indicated a limited capability of most systems to meet project criteria. Promising results (200 to 600-fold concentration and up to 88% recovery of bacteria) were obtained using hollow fiber concentration systems modified to incorporate repeated backwash steps.

Due to manpower constraints, Goddard Space Flight Center had to discontinue projects not directly related to the space mission and was unable to continue evaluation and development of a satisfactory concentration system to go with the flow system for the ATP assay.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water and a spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment and management of waste water and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies and to minimize the adverse economic, social health and aesthetic effects of pollution. This publication is one of the products of that research, a most vital communications link between the researcher and the user community.

The research reported herein was directed toward developing a rapid, automatable method for quantitation of total viable bacteria in potable drinking water, based on the firefly luciferase ATP assay. The application of this procedure as a potable water quality monitoring tool would provide real-time assessment of bacterial levels in treated distribution water, compared to standard bacterial testing that requires 24 to 48 hours to obtain results. The study reports that the ATP assay was adapted to a flow system allowing completion of assays in less than two minutes. Various filtration systems were evaluated for concentrating bacteria from treated water to achieve the minimum sensitivity level necessary for the assay.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

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PREFACE

Present standard methods for the examination of bacteriological quality of water supplies are dependent on time-consuming culture processes. Efficient control of contamination break-throughs into finished waters or detection of water quality deterioration in distribution networks is delayed until results from the traditional coliform procedure and standard plate counts can be made available. Maintenance and control of water quality would be greatly enhanced by the availability of a method for rapid measurement of bacterial content in water supplies.

The National Aeronautics and Space Administration (NASA) has supported the development of bacterial detection systems through research undertaken by the Goddard Space Flight Center (GSFC) near Washington, DC, and the Johnson Space Center (JSC) in Houston. GSFC has developed the expertise to detect and estimate concentrations of viable bacteria in fluids using luminescent assays, particularly the firefly luciferase assay for adenosine triphosphate (ATP). At JSC these assays have been adapted for an ongoing project to develop a series of analytical tests for wastewater effluent. Through an inter-agency Memorandum of Understanding between NASA and the U.S. Environmental Protection Agency (EPA) the two centers have collaborated and developed, as documented in this report, a rapid, automatable system applicable to the measurement of bacteria in drinking water supplies.

The system described in this report is not intended to replace standard techniques for measuring fecal or total coliforms but rather to offer a means of determining total biological load in "real time" for more useful monitoring activities. The ATP assay actually detects the presence of all living microorganisms in the water sample. If the majority of the organisms present are assumed to be bacteria, an ATP assay can be directly related to the concentration of total viable bacteria. The significance and utility of a system that measures total microbial or bacterial biomass in this manner will have to be established through field tests and laboratory studies that correlate results with standard techniques. Due to manpower constraints, GSFC has had to discontinue projects not directly related to the space mission, and will be unable to perform the needed correlation studies. Because of the potential value of this system in the area of water quality, we feel this research should be continued elsewhere.

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ABSTRACT

This research program was initiated to develop a rapid, automatable system for measuring total viable microorganisms in potable drinking water supplies using the firefly luciferase ATP assay.

The ATP assay was adapted to an automatable flow system that provided comparable sensitivity with established methodology (10^5 bacteria per ml) and assays in less than two minutes. Quality controls for required reagents were established. To achieve the sensitivity necessary for bacterial measurements in water, the sample must be concentrated prior to assay. Filtration systems were evaluated for ability to concentrate bacteria from large sample volumes rapidly, efficiently, and without damage to the organisms.

Results indicated a limited capability of most systems to meet project criteria. Promising results (200 to 600-fold concentration and up to 88 percent recovery of bacteria) were obtained using hollow fiber concentration systems modified to incorporate repeated backwash steps.

This report was submitted in fulfillment of EPA-IAG-D6-0982 and a Memorandum of Understanding between NASA and EPA, MOU-2, by Goddard Space Flight Center under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period April 15, 1976, to April 15, 1977, and work was completed as of April 15, 1977.

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ACKNOWLEDGEMENT

The cooperation of the Johnson Space Center in the development of concentration systems is gratefully acknowledged. We are particularly indebted to Eldon Jeffers of the Boeing Company, Houston, Texas, for his contributions in the design and testing of these systems.

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SECTION 1

INTRODUCTION

Intracellular ATP extracted from microorganisms in drinking water supplies can be detected and quantitated using the firefly luciferase ATP assay. This assay is a rapid, "wet chemical" reaction that is easily automated. Relative ATP concentrations could be used to monitor changes in the levels of microbial populations, detecting contamination break-throughs into finished water supplies or water quality deterioration in network distribution systems in "real time". If the majority of microbes in the water supply are assumed to be bacteria, ATP levels could be used further to estimate the total number of bacteria present. It has been observed that the deviation of ATP content of bacteria, taking into account differences between species and over the growth cycle, does not exceed one order of magnitude.

The sensitivity limit of the ATP assay with laboratory cultures of pure bacterial strains is about 10^5 bacteria per ml. If 500 bacteria per ml is considered the acceptable upper limit for non-coliform bacteria in drinking water supplies, contaminating levels of bacteria must be concentrated by a factor of at least 200 to permit detection by an ATP assay. For example, the volume of a 20 liter sample containing 500 bacteria per ml would have to be reduced to 100 ml with complete retention of bacteria to provide a concentration at the sensitivity limit of the assay. In order to insure operation above the minimum sensitivity limit, increased bacterial concentrations are desirable.

The present study has focused on the development of an automated ATP assay and a suitable technique for concentrating low levels of bacteria from large sample volumes for subsequent analysis. Criteria for an automated assay included sensitivity comparable to established methodology, minimum mechanical manipulations, real time analysis, and quality controls for required reagents. The concentration technique had to be compatible with automation and provide a high concentration factor, a rapid concentration rate, and complete recovery of intact, undamaged bacterial cells from large sample volumes in a reduced volume that could be diverted to an automated ATP assay system.

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SECTION 2

CONCLUSIONS

The ATP assay has been adapted to a semi-automated flow system in which sample and reagents are proportioned automatically using tubing and peristaltic pumps. A sensitivity of about 10 bacteria per ml has been established using dilutions of Escherichia coli in deionized water. The entire process of ATP acid-extraction, dilution, and assay requires less than two minutes. Quality controls for reagents, particularly the luciferase-luciferin enzyme preparation and chemical ATP standard, have been established.

Development of an appropriate technique for concentrating bacteria rapidly and efficiently from large volumes of drinking water has involved modifications and extensive testing of various concentration systems. Continuous flow centrifugation provided satisfactory results but was rejected as an appropriate technique due to incompatibility with automation. Direct extraction of organisms concentrated to near dryness on a filter membrane surface in an in-line system proved inefficient. Filtration systems for processing large sample volumes using tangential or radial flow across a flat membrane surface are commercially available for concentration of macrosolutes and in some cases viruses. Attempts to adapt these systems to bacterial concentration were generally unsuccessful. Bacterial recoveries in the final reduced sample volumes, even with backwashing, were incomplete and unreliable. Concentration rates and factors were limited.

Test results using hollow fiber membrane filtration systems adapted for bacterial concentration have been very promising. Using a compact hollow fiber system that provides considerable membrane surface area, large volumes of water can be processed rapidly through the pores of the fibers. Bacteria concentrated on the inner surfaces of the fibers can be resuspended in a reduced volume of sterile, deionized water by backwashing. The result is a system that provides acceptable concentration factors, rates, and bacterial recoveries.

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The complete system, hollow fiber concentrator coupled with ATP flow system, as tested with E. coli-seeded deionized water samples, preliminary meets the overall objective of this research project in providing a rapid, automatable means to detect contaminating levels of microorganisms in potable water supplies.

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SECTION 3

RECOMMENDATIONS

The proposed system for rapid concentration and detection of microorganisms in drinking water supplies is based largely on data obtained with E. coli-seeded deionized water samples. The system remains to be challenged by other bacterial isolates from water supplies and by actual drinking water samples that may or may not be contaminated. At the Johnson Space Center in Houston a similar system has proven successful in measuring microbial populations in wastewater effluent. However, the considerably higher levels of microorganisms found in effluent are easier to concentrate and detect than the very low levels in potable water. Success with drinking water samples remains to be demonstrated experimentally and cannot necessarily be extrapolated from the Johnson Space Center results.

The ultimate significance and utility of a method that provides an immediate measure of total viable microorganisms in terms of intracellular ATP content will also have to be determined with field tests. Correlation studies with standard techniques for determining total bacterial content will be critical in evaluating ATP results, but limitations of standard techniques should not be overlooked in the process. The potential value of an automated method for "real time" monitoring of microbiological load in potable water supplies may well outweigh any lack of specificity for bacterial populations per se.

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SECTION 4

FIREFLY LUCIFERASE ATP ASSAY SYSTEM

Use of the firefly luciferase ATP assay to quantitate bacteria concentrated on a filter or in a fluid sample involves two basic steps, the complete extraction of ATP from the organisms and the reaction of that free ATP with the luciferin-luciferase enzyme mixture to produce light. Comparison of these light units with light produced by a known concentration of standard ATP permits the determination of the concentration of extracted ATP. Because the ATP content of bacteria is reasonably constant from species to species[1] and through a growth cycle[2-5], the direct correlation between light production and extracted ATP can be further extrapolated to bacterial concentration in the sample tested.

ATP FLOW SYSTEM

Established methodology for measuring ATP extracted from a bacterial suspension generally includes the addition of extractant by pipet to the sample in a polypropylene test tube, dilution by pipet, intermediate mixing using a vortex instrument, distribution of the enzyme mixture into individual cuvettes by pipet, and final injection by tuberculin syringe of 0.1 ml extracted, diluted sample into a luciferase cuvette positioned in front of a photomultiplier tube.[6] The firefly luciferase ATP flow system greatly reduces the need for such mechanical manipulations: a configuration of interconnected tubes through which sample and reagents are pumped provides for automatic and appropriately timed extraction, dilution, and assay.[7] If the ATP is extracted directly from bacteria concentrated on a filter surface, the flow system can be abbreviated to include only diluent and the enzyme mixture.

INSTRUMENTATION

The complete ATP flow system is schematically diagrammed in Figure 1. Buchler peristaltic pumps, operated at a high speed (5), initiate and sustain the flow of sample and reagents in the tubes. Relative flow

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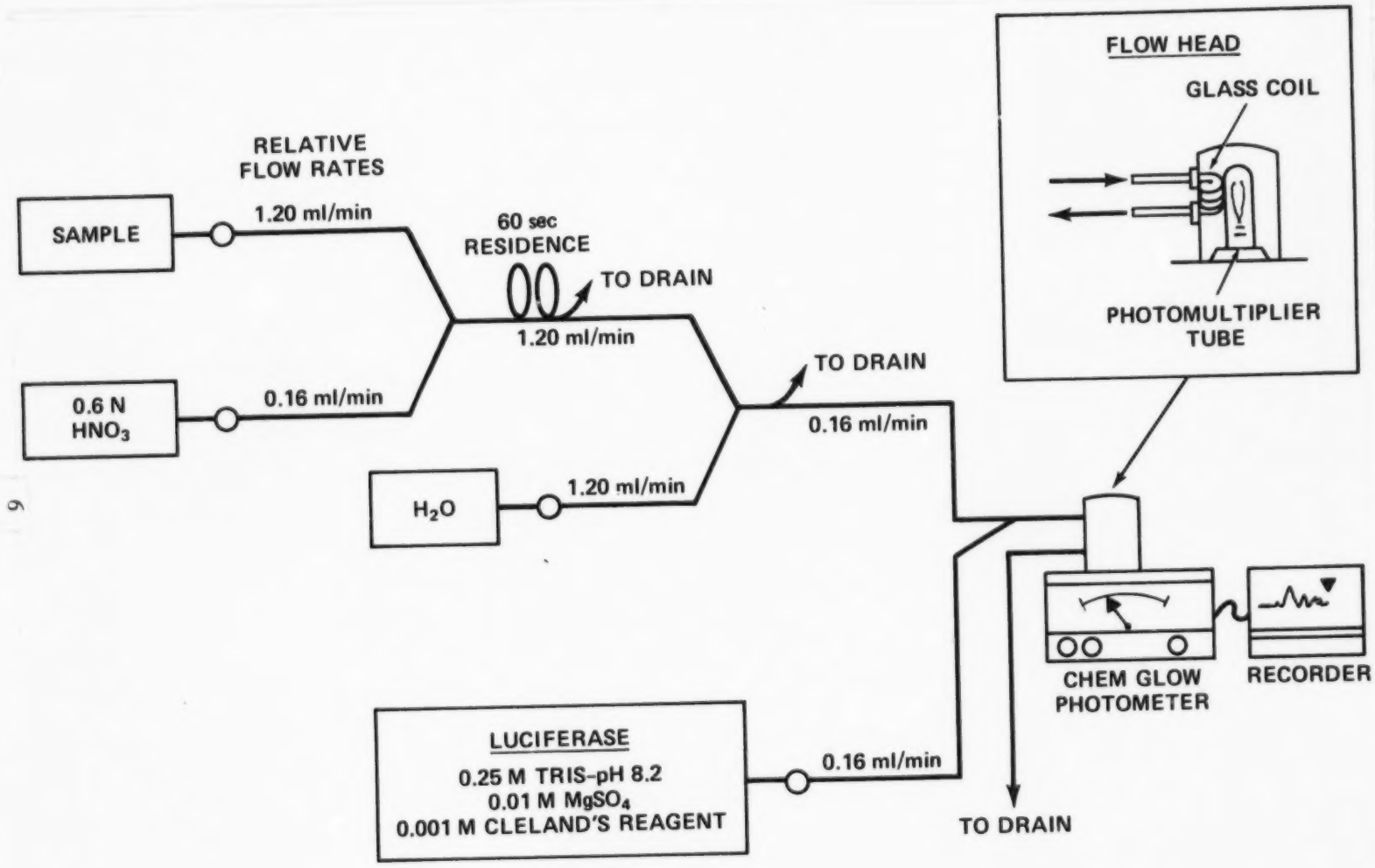

Figure 1. Schematic of automated firefly luciferase flow system for detecting bacterial ATP including nitric acid extraction and subsequent dilution.

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rates as noted in Figure 1 are designed to provide the optimal concentration of nitric acid extractant (0.1 N), with minimal sample dilution (50%), and a conservative volume of luciferase enzyme preparation (0.2 ml). The luciferase enzyme is pulsed into the flow system only as the final processed sample reaches the photomultiplier tube.

Complete extraction of bacterial ATP occurs in a sixty second residence time of sample with 0.1 N nitric acid, made possible by an extra length of tubing prior to diluent entry. The extracted, diluted ATP sample and the luciferin-luciferase enzyme mixture (one vial Dupont enzyme rehydrated with three ml of 0.25 M TRIS-pH 8.2, 0.01 M $MgSO_4$, 0.001 M Cleland's reagent) pass through needle constrictions which increase flow velocities for better mixing. The reaction mixture immediately enters a glass coil positioned next to a photomultiplier tube. A Chem Glow photometer (American Instrument Company) equipped with a coiled flow cell was used in this project to measure maximum light output as the reaction mixture flows through the coil. An Aminco Integrater-Timer was also tested to integrate the area under the light reaction curve and measure the total light production.

TESTING PROCEDURES

Sensitivity and linearity of the ATP assay as incorporated in the described flow system were determined using known concentrations of E. coli (determined by plate counts of overnight cultures of E. coli ATCC 25922) diluted in saline (0.9% NaCl) and tap water dechlorinated with 53 mg thiosulfate per liter. Sample light output was considered significant if higher than background light emission. A blank consisting of sterile, deionized water was processed to determine background light signal, which was routinely subtracted from the sample and ATP standard response. Sample ATP concentrations were determined by comparison with standards (0.01 µg ATP/ml), prepared by diluting purified, chemical ATP (Sigma Chemical Company) in sterile, deionized water.

A conversion factor of 2.5×10^{-10} µg ATP per cell was used to calculate bacterial numbers. This factor represents the average ATP content of 19 bacterial species. The intracellular ATP levels ranged from 0.28 to 8.9×10^{-10} µg.[1] ATP content may also vary depending on the growth phase and culture conditions.[2-5] For final application to potable water supplies, the conversion factor should be determined using bacterial isolates from actual water samples.

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SENSITIVITY

The light output of the luciferase reaction can be measured in terms of maximum intensity (peak height) or total light flux emitted over a fixed time period (integration).[8] A 20 second integration of the light reaction curve when compared to peak height affords improved sensitivity, but values obtained for lower concentrations of bacteria are condsidered unreliable due to high standard deviations.[7] All data presented in this report are based on peak height measurements.

Figure 2 indicates the results of a typical E. coli concentration curve generated by the described testing procedures. The system is linear from 10^5 to 10^9 bacteria per ml in either saline or tap water. The correlation coefficient for linear regression analysis is 0.9923 and the slope is not significantly different from one at the 5% level. The lower sensitivity limit of 10^5 cells/ml is equivalent to that achieved by established ATP methodology.

REAGENTS AND QUALITY CONTROLS

Extractants

A wide range of extracting agents have been investigated previously for use in the ATP assay injection system including inorganic acids such as nitric (HNO_3), perchloric ($HCLO_4$), and sulfuric acid (H_2SO_4), ionizing organic solvents such as dimethylsulfoxide (DMSO), and boiling buffer (TRIS).[1] Of these extractants nitric acid demonstrates superior extraction efficiency for bacterial ATP.[9] Accordingly, nitric acid has been optimized for use in the flow system: extraction is efficient and tubing used in the system is acid-resistant and not affected over long periods of time. When an acid is used as the extracting agent, however, the luciferase enzyme system must be appropriately buffered to maintain the optimal reaction pH of 7.75. Dilution of the sample with sterile, deionized water after extraction and rehydration of the luciferase powder in an appropriate molarity of TRIS buffer maintains the proper pH-8.

Luciferin-Luciferase Enzyme Mixture

The firefly luciferase ATP assay involves the specific reaction of ATP with luciferin-luciferase extracted from dessicated firefly lanterns. Highly purified luciferase requires the addition of synthetic luciferin which inceases the cost of the assay but improves sensitivity 100-fold. In our study, we have used purified luciferase provided by E. I. Dupont de Nemours and Company, Inc., or luciferase prepared in our own laboratory.[10]

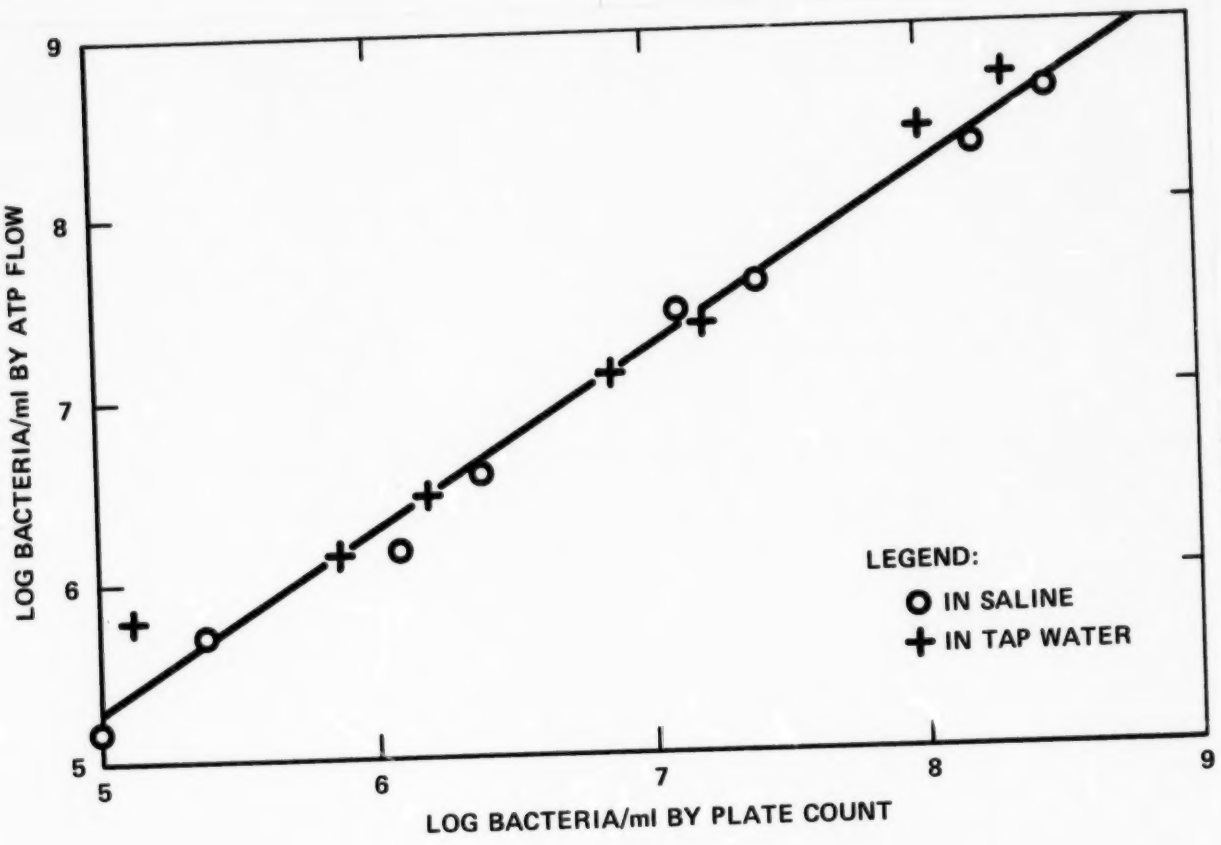

Figure 2. Concentration curve of E. coli in saline and in tap water: bacteria/ml by ATP flow vs. plate count

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With nitric acid as the extractant, purified luciferase is rehydrated in 0.25 M TRIS buffer (three ml per vial of Dupont enzyme) with the necessary enzyme cofactor magnesium at a concentration of 0.01 M $MgSO_4$. The addition of 0.001 M Cleland's reagent (dithiothreitol) in the TRIS buffer promotes enzyme stability by protecting sulfhydryl groups. Rehydrated enzyme stored in the dark at 10°C will remain stable (negligible activity loss) for four hours (Table 1); with the addition of Cleland's reagent, high activity is maintained for eight hours (Table 2). Pooled enzyme is prepared in the minimum quantity needed based on the number of samples or experiments to be run that day. If enzyme use surpasses eight hours the addition of 0.001 M EDTA (ethylene diamine tetraacetate) in the TRIS buffer will preserve functional activity for about two days (Table 2). As in the case with enzymes in general, precautions must be taken to avoid denaturation due to overheating or exposure to marked temperature fluctuations. The enzyme preparation should be brought to 20°C, the optimal reaction temperature, before assaying a sample.

TABLE 1. STABILITY OF LUCIFERIN-LUCIFERASE ENZYME MIXTURE: PERCENT INITIAL ENZYME ACTIVITY

Hours After Hydration*	25° C/Light	25° C/Dark	10° C/Dark
2	71	87	96
4	41	78	94
6	18	62	76
8	11	65	84
12	2	45	66

* 1.5 ml 0.25 M TRIS, pH 8.2, per vial Dupont enzyme.

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TABLE 2. STABILITY OF LUCIFERIN-LUCIFERASE ENZYME MIXTURE: PERCENT INITIAL ENZYME ACTIVITY WITH CLELAND'S REAGENT AT 10°C IN THE DARK

Hours After Hydration*	With 0.001 M EDTA	Without EDTA
8	100	93
23	97	73
48	66	64
72	55	55
96	49	44

* 1.5 ml 0.25 M TRIS, pH 8.2, per vial Dupont enzyme.

The luciferase mixture emits low levels of light without the addition of any ATP. This inherent light may be due to small amounts of ATP in the enzyme mixture that are not removed during purification. Incubation of the rehydrated enzyme at room temperature for at least half an hour prior to assay will reduce the level of inherent light considerably (Table 3).

TABLE 3. DECAY OF INHERENT LIGHT WITH TIME

Minutes After Hydration*	Inherent Light (relative light units)	% Initial
0	4.71×10^6	100
1	3.90×10^6	83
2	3.06×10^6	65
3	2.37×10^6	50
4	1.98×10^6	42
5	1.62×10^6	34
10	8.16×10^5	17
15	5.49×10^5	12
20	4.11×10^5	9

* 1.5 ml 0.25 M TRIS, pH 8.2, 0.01 M $MgSO_4$, 0.001 M Cleland's per vial Dupont enzyme.

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Stability of Chemical ATP

Because inherent light and enzyme activity may differ between preparations of luciferase batches, and may be effected by environmental conditions such as heat, light, pH, foaming, etc., a standard solution of purified chemical ATP should be measured periodically for quality control. Chemical ATP (Sigma Chemical Company) remains stable at room or refrigerated temperatures for at least 48 hours when prepared in 0.001 M EDTA and 0.01 M $MgSO_4$ (Table 4). Variations in manufacturer's lot preparations of purified ATP should be taken into account for routine use of an ATP standard, either as a daily quality control or as a means of determining sample ATP concentrations.

TABLE 4. STABILITY OF CHEMICAL ATP: PERCENT INITIAL ACTIVITY OF ATP STANDARD

Hours After Hydration	With EDTA/Mg*		Without EDTA/Mg	
	10° C	25° C	10° C	25° C
3	87	89	70	74
7	94	98	73	61
24	93	88	54	17
48	95	95	36	<1

* ATP hydrated in 0.001 M EDTA, 0.01 M $MgSO_4$.

Stability of Bacterial ATP

Bacterial ATP extracted in 0.1 N nitric acid is subject to hydrolysis if allowed to stand in the presence of the acid for any length of time. If the acid-extracted ATP from E. coli is diluted fifty percent with sterile, deionized water, a maximum of only thirty percent of the bacterial ATP will hydrolyze over a twelve hour period. The ATP flow system provides dilution of the sample after sixty seconds and subsequent reaction with the luciferase enzyme system in a matter of seconds, thus avoiding any significant loss of sample ATP by hydrolysis.

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SECTION 5

BACTERIAL CONCENTRATION PROCEDURES

GENERAL CRITERIA

In order to detect contaminating levels of microorganisms in potable water supplies using the firefly luciferase ATP assay, the organisms first must be concentrated by a factor of at least 200, and preferably more to ensure operation above the minimum sensitivity limit of the assay. If the organisms are concentrated on a membrane filter surface, they must be recovered, intact and undamaged, in a small volume of water (backwash, for example) for subsequent introduction into the ATP flow system. Although direct acid extraction of ATP from organisms on a filter is a possibility, the recovery of viable cells in a small volume of water enables confirmation of ATP assay results by standard plate counts, Coulter counts, and other bacterial detection methods available in this laboratory (luminol iron porphyrin assay, photobacterial FMN assay, and Bactec).

Effective concentration of organisms in the sample is dependent on the final volume in which they are collected or resuspended. For example, 10 liters reduced to 10 milliliters provides 1000-fold concentration, if all bacteria are recovered in the final volume. In any case, an acceptable concentration technique has to be able to process large sample volumes (at least 10 liters) in a period of time compatible with the desired frequency of sampling, without exposing organisms to conditions of stress that would significantly alter internal ATP content or damage cells structurally.

To our knowledge there is no concentration technique commercially available at present that presumes to meet the above criteria. Consequently, we have attempted to adapt numerous techniques designed for concentration of macrosolutes, and in some cases viruses, to the concentration of bacteria [1] for the purposes of this project.

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TESTING PROCEDURES

Each concentration technique was challenged to concentrate bacteria from sterile, deionized water samples seeded with a known concentration of E. coli. The percent bacteria recovered in the final concentrate was determined by measuring bacterial levels before and after concentration by means of plate counts, ATP assays, Coulter counts, or luminol iron porphyrin assays,[7] or a combination of these enumeration methods. Concentration factors were based on absolute reduction of sample volume. Ideal test results would include greater than 200-fold concentration with 100% recovery of bacteria. If, after a minimum of three test runs, a concentration factor of ten could not be achieved with recovery of at least 50% of the bacteria, the particular concentration technique was abandoned.

CENTRIFUGATION

Continuous flow centrifugation (Sorvall) allowed processing of large sample volumes with adequate bacterial recovery, approximately 75%, but very low concentration rates. Manipulations involved in collecting the bacterial concentrates precluded automation of the technique, and centrifugation was not pursued further.

DIRECT IN-LINE FILTRATION

A simple, automatable method for directly concentrating bacteria on a membrane filter is diagrammed in Figure 3.

Sample under pressure was passed through a 0.45 µm cellulose acetate membrane filter housed in a Swinnex in-line filter unit (Millipore Corporation). Bacteria concentrated directly on the filter surface were found to be impacted so strongly as to resist effective backwashing. Substitution of different filter types did not alleviate the problem. Nuclepore polycarbonate membranes (a membrane type whose pores should not entrap bacteria as readily as cellulose or fibrous filter types) were tried but an effective seal could not be maintained.

Attempts at direct extraction of bacterial ATP by backwashing with nitric acid instead of sterile, deionized water were also unsuccessful. Table 5 indicates results with in-line extraction versus external extraction, where the loaded filter was removed from the holder and immersed in acid, a standard procedure for marine biomass determinations.[12] With ten-fold concentration an average of only 18% recovery was achieved.

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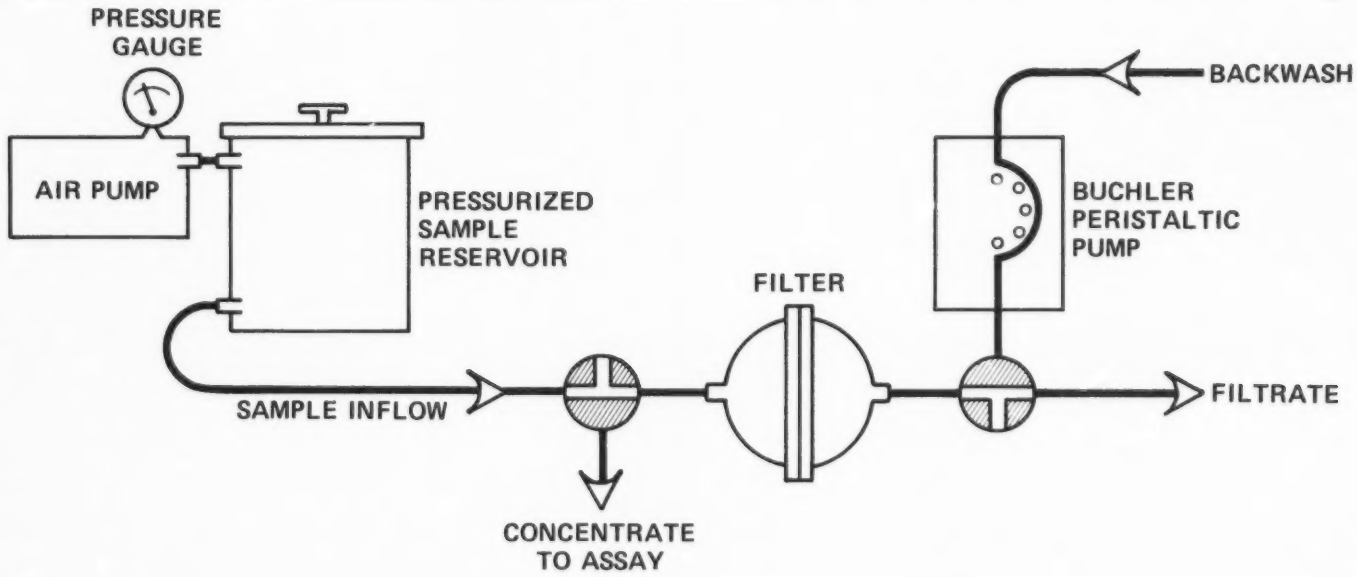

Figure 3. Schematic of Millipore Swinnex in-line filter using backwash. System is diagrammed in concentrating mode; valves are rotated 90° for backwashing and assay.

TABLE 5. TEST RESULTS USING MILLIPORE IN-LINE FILTER CONCENTRATOR WITH IN-LINE EXTRACTION

Sample	In-Line Extraction (μg ATP/ml)	External Extraction (μg ATP/ml)	% Recovery with In-Line Extraction
1	3.3×10^{-4}	1.9×10^{-3}	17
2	3.5×10^{-4}	1.85×10^{-3}	19
3	2.5×10^{-4}	1.8×10^{-3}	14
4	8.1×10^{-5}	1.08×10^{-3}	7
5	1.0×10^{-4}	3.3×10^{-4}	30

FLAT SURFACE MEMBRANE FILTRATION

Alternatives to direct filtration using flow forces perpendicular to the membrane surface are flat surface membrane filtration systems that operate by radial or tangential flow, promoting high flow velocities parallel to the membrane surface. Parallel flow maintains shear forces that reduce particle buildup on the membrane. Concentration is achieved by volume loss through the filter and retention of particles in a decreased sample volume. When such systems were tested with bacterial samples, recovery of the organisms in the final retentate was limited, i.e., less than 50%, since some bacteria remained on the filter surface, perhaps because of vertical forces created by volume loss through the filter. Addition of a backwash step was required in all cases in order to improve bacterial recoveries.

Uni-Pore Radial and Stirred Flow Cells (Biorad Laboratories)

Less than 50% bacterial recoveries were obtained with the Radial Flow Cell, which relies on radial flow to keep particles in suspension, or with the Dual Membrane Stirred Cell, which relies on a Teflon-coated magnetic stir bar set in motion above the filter. Bacterial leakage past O-rings was a continual problem that affected consistency of results, regardless of whether the filter was polycarbonate, cellulose acetate, or polyvinylchloride copolymer.

Sartorius Ultrafiltration System (Sartorius Corporation)

The Sartorius Ultrafiltration System design is well adapted for processing large sample volumes since a large surface area is provided by a stack of up to 15 membrane filter plates (170 cm^2 each). The recommended macrosolute concentration procedure involves sample recirculation across the plates by tangential flow and collection of the concentrate in a reduced volume. This has not proved to be an acceptable method for concentrating bacteria. With each pass of the retentate over the filter surface(s), organisms were trapped and lost. Although the bacteria may not have been attached tightly to the membrane, they could not be recovered simply by sample recirculation. As diagrammed in Figure 4, a backwashing step was necessary to recover bacteria from the filter surface.

With 10-fold sample concentration, bacterial recoveries were improved markedly, as shown by test results in Table 6, by treating the sample with 0.001% Triton X-100 (a nonionic detergent) or 0.1% rhozyme (a mixture of proteases and glucosidases extracted from Aspergillus oryzae), and by substituting Nuclepore polycarbonate filters for the recommended cellulose nitrate filters. Both Triton X-100 and rhozyme had been shown to enhance filtration without effecting bacterial ATP in other applications.[13]

TABLE 6. TEST RESULTS USING SARTORIUS ULTRAFILTRATION SYSTEM WITH TANGENTIAL FLOW AND BACKWASH

Concentration Factor*	% Recovery	Filtration Aid
2	79	--
4	63	--
10	21	--
10	90	With TX
10	92	With rhozyme
5	50	Cellulose acetate filter, TX
5	80	Polycarbonate filter, TX

* Each factor represents a separate test.

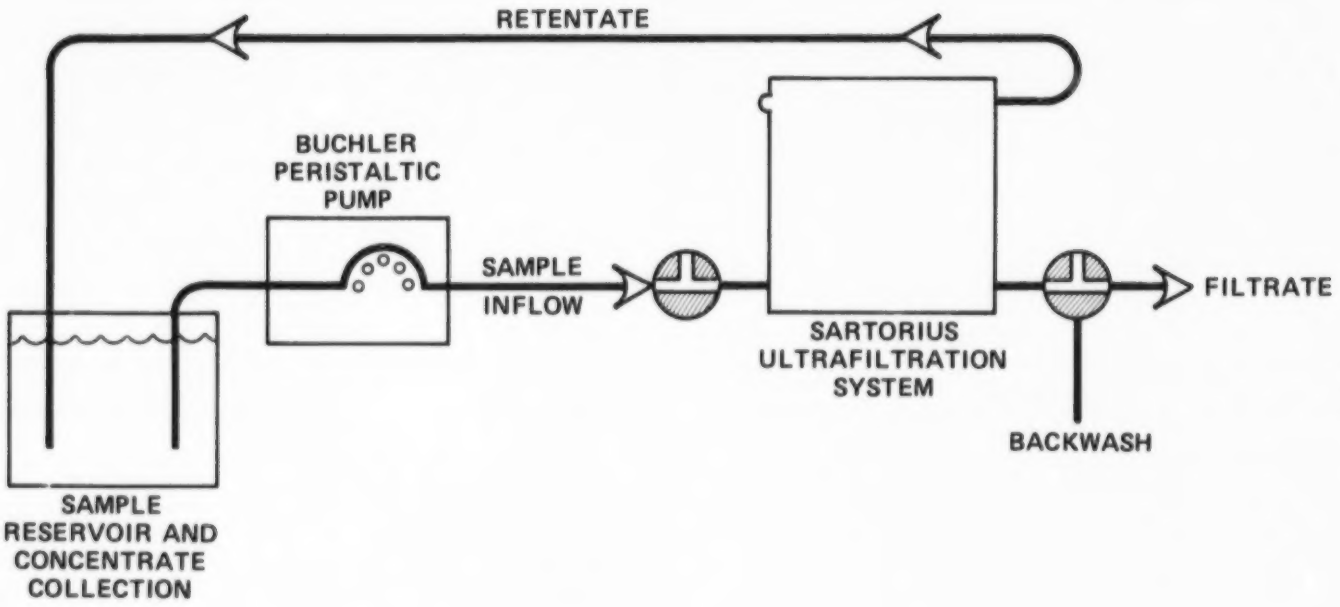

Figure 4. Schematic of Sartorius Ultrafiltration System using tangential flow and backwash. System is diagrammed in concentrating mode; valves are rotated 90° for backwashing provided by pressurized tap source of sterile, deionized water.

Attempts to increase the concentration factor to 40 resulted in bacterial recoveries as low as 12%. Excessive quantities of backwash, negating concentration effect, were required to recover a majority of the organisms. Backwash flow across the flat membrane surfaces was probably not uniform, with the result that some areas were not flushed when the backwash volume was limited. These results are in accord with those obtained independently at the Johnson Space Center.

Pellicon Cassette Molecular Filter (Millipore Corporation)

The configuration of this high volume molecular filtration cell is very similar to that of the Sartorius Ultrafiltration System in the use of membrane filter plates or "cassettes" that provide a large square surface area. With 50-fold concentration 40 to 90% recoveries were typical, although recoveries of less than 10% occurred randomly. Use of Triton X-100 made no significant improvement. As with the Sartorius system, configuration probably limited overall performance with bacterial concentration and backwashing.

HOLLOW FIBER MEMBRANE FILTRATION

Further testing of flat membrane filtration methods was suspended in favor of hollow fiber concentration systems which use parallel flow forces but with a different membrane configuration. In these systems the sample is pumped through a set of hollow fibers, each fiber being a uniform membrane cylinder whose inner side is exposed to the sample stream. The fibers contain pores of controlled dimensions such that particles larger than a given molecular weight are retained in the sample stream while smaller molecular weight solutes pass through the membrane in the filtrate. Particle concentration occurs by volume reduction as water filters through the fiber pores. The recommended procedure for macrosolute concentration involves recirculating the sample through the fibers and collecting a reduced volume of concentrated solution. As with flat surface membrane systems, backwashing proved necessary when attempting to concentrate and recover bacteria.

Bio-Fiber 80 Miniplant (Biorad Laboratories)

The first hollow fiber concentration system tested was the Bio-Fiber 80 Miniplant which provides a surface area of 10,000 cm^2 using cellulose acetate fibers of 30,000 M.W. cutoff.[14,15] Results of experimental tests in Table 7 indicate that with repeated backwashing (and volume reduction

between each backwash step) up to 83% of the bacteria were recovered at concentration factors from 15 to 250. Although the Bio-Fiber Miniplant appeared to meet the overall concentration requirements of this project, it is no longer commercially available.

TABLE 7. TEST RESULTS USING BIO-FIBER 80 MINIPLANT WITH 15 TO 250-FOLD CONCENTRATION

Test Procedure	% Recovery $\pm \sigma$*
Recirculation	42 $\pm$ 17
Backwash only	64 $\pm$ 23
Backwash, refilter, backwash	81 $\pm$ 13
Backwash, refilter, backwash, refilter, backwash	83 $\pm$ 10

* Standard deviation calculated on the basis of ten test runs.

Diaflow Hollow Fiber Concentrators (Amicon Corporation)

Results with Amicon hollow fiber concentrators were as promising as those with the Biorad Miniplant. Preliminary tests with a small Amicon cartridge, providing a filter surface area of 1,000 cm^2 and a M.W. cutoff of 100,000, indicated it was possible to achieve by recirculation alone close to 100% recovery of bacteria with 10-fold concentration. Increasing the concentration factor to 100, however, reduced recoveries to as low as 32%. Use of a dual cartridge adapter, fitted with two 50,000 M.W. hollow fiber cartridges, increased concentration rates so that a ten liter sample could be reduced to 100 ml in less than 50 minutes; however, bacterial recoveries were inconsistent, ranging from 43 to 100%.

Based on the assumption that recoveries were low and inconsistent because of accumulation of bacteria on the fiber walls, the system was modified to allow a backwash of sterile, deionized water as diagrammed in Figure 5. The bacteria were concentrated on the inner walls of the fibers directly rather than by recirculation. The principle of tangential flow was still employed for a more "gentle" impact effect. Air and water under pressure entered the previously capped port and filtrate port, respectively, to flush out bacteria for collection through the original sample influx port.

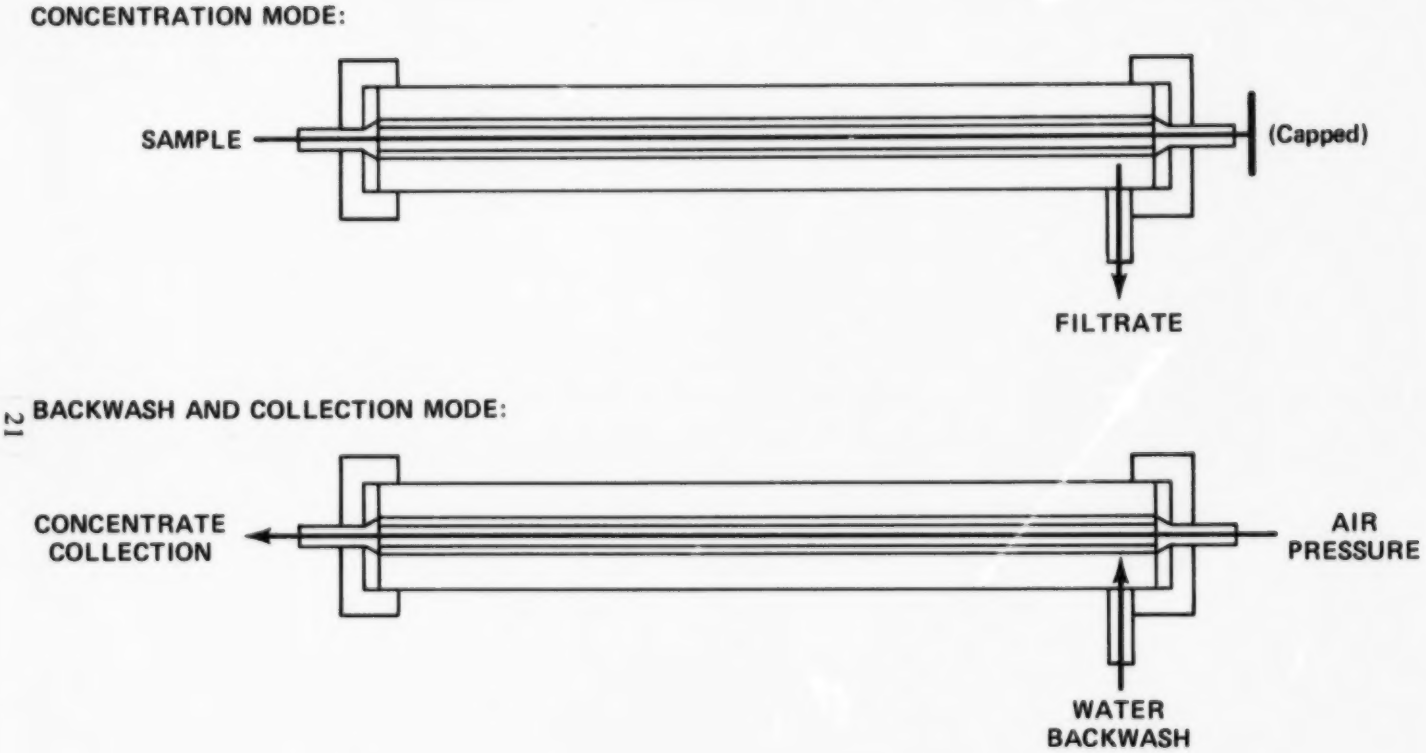

Figure 5. Schematic of Amicon hollow fiber cartridge modified for backwash capability

Test results using this setup are given in Table 8. Bacterial recoveries using the small Amicon cartridge were excellent (95%), but concentration rates and factors were limited. Use of the large Amicon cartridge with 10,000 cm^2 filter surface area provided the most promising results. At concentration factors as high as 600, a single backwash step resulted in 53% recovery of bacteria. This percentage was increased substantially to 88% with an additional backwash step. The initial 100 ml backwash was refiltered before the second backwash was introduced into the cartridge so that extra backwashes would not increase final concentrate volume or limit the maximum concentration factor.

TABLE 8. TEST RESULTS USING AMICON HOLLOW FIBER CARTRIDGE WITH BACKWASH

Cartridge Size Filter Area (cm^2)	Maximum Concentration Factor	Test Procedure	% Recovery $\pm\sigma$*
1,000	60	Backwash	95 $\pm$ 5
10,000	600	Backwash	53 $\pm$ 21
		Backwash, refilter, backwash	83 $\pm$ 14
		Backwash, refilter, backwash, refilter, backwash	88 $\pm$ 12

* Standard deviations calculated on the basis of ten test runs.

DISCUSSION

Hollow fiber concentrators modified for backwash capability proved to be the only concentration systems tested that allowed greater than 200-fold concentration of bacteria with adequate recovery of the organisms (88%). The fibers supplied by Amicon are composed of non-cellulosic polymers which should be durable for (a period of) months. Tests for leaks or breaks in the filter material using blue Dextran 2000 dye confirmed the integrity of the fibers used at GSFC. A thirty minute rinsing procedure using sterile, deionized water has been effective during the testing period in minimizing a buildup of bacteria in the fibers between samples. A routine rinse with 0.1 N NaOH followed by sterile, deionized water may prove necessary for

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long term use of the filtration system. Although extended testing of an Amicon setup using actual drinking water samples is needed before a final recommendation can be made, the hollow fiber concentrator modified as described seems to meet proposed criteria for concentrating bacteria for subsequent ATP analysis.

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